Expanding the physiological roles of Multiple Peptide Resistance Factor: MprF function in *Bacillus subtilis*

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Abstract

Multiple peptide resistance factor (MprF) is a membrane bound protein that adds lysine to phosphatidylglycerol (PG) using an elongator Lys-tRNA^{Lys}. This lipid modification changes the net charge of the cell wall which results in cellular permeability changes that confer resistance to cationic antibiotics. Point mutations in *mprF* have been shown to cause spontaneous resistance to daptomycin, a last resort antibiotic in the treatment of the pathogen Methicillin resistant *Staphylococcus aureus* (MRSA) that is believed to have CAMP-like properties and mode of action [1]. Due to the effects of altering membrane permeability and increased antibiotic resistance, *mprF* constitutes as a virulence factor in MRSA. Cell wall modifications by MprF have been shown to have broader effects in other bacteria such as the Gram-negative *Pseudomonas aeruginosa* where it confers resistance to acidic conditions, chromium, cefsulodin, and protamine. In this work expanded roles of *mprF* in the Gram-positive microorganism *Bacillus subtilis* were elucidated through Biolog Phenotype MicroArrays, the technology of which allows for the performance of high-throughput screens of 2,000 physiological phenotypes. Presented here is data based on the comparison of wild type *B. subtilis* 1A100 growth to that of a marker-less deletion strain of *mprF*. This data shows a decreased ability of the deletion strain to survive in the presence of several antibiotics, cationic molecules and lipophilic compounds indicating a global effect of *mprF* on cell wall permeability in *B. subtilis*. These effects, while consistent with previous data from *L. monocytogenes*,

indicate that MprF plays similar, yet varied, roles in diverse microorganisms through modification of cell wall properties. The discovery of such a widespread mechanism among bacterial species has important implications for the development of an MprF inhibitor that has broad range specificity to a multitude of pathogens.

Introduction

A common target exploited by many antibacterial host defense mechanisms is the highly anionic property of the bacterial cell wall. The highly negative components of bacterial membranes such as phospholipids, teichoic acids, and lipid A moieties are targeted by positively charged antimicrobial peptides produced by human neutrophils, plants, fungi and other bacterial species. Bacteria have developed multiple mechanisms to evade these cationic antimicrobial peptides (CAMPs), one method of which is neutralization of the net negative charge of the cell wall by modifying negatively charged components. Multiple peptide resistance factor (MprF) modulates the charge of the cell membrane by transferring an amino acid (aa) from a cognate aminoacyl-tRNA (aa-tRNA) to a free hydroxyl group of phosphatidylglycerol (PG) (Figure 1). MprF is an integral

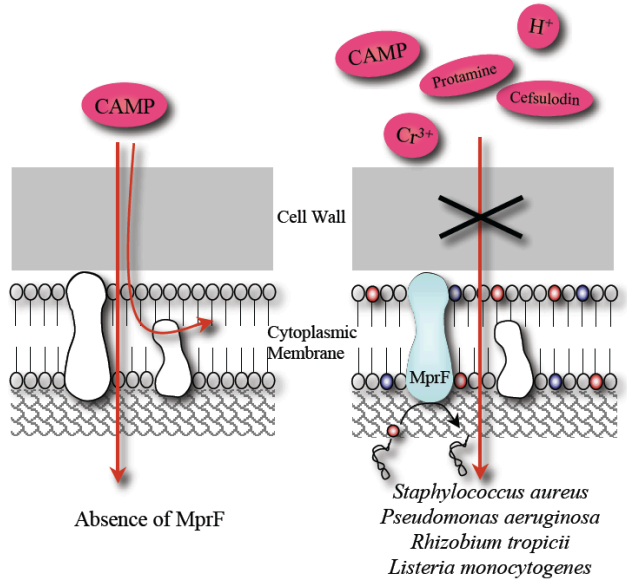

Figure 1. Phenotypes conferred by the presence of MprF. MprF utilizes a cognate Lys-tRNA^{Lys} (red) in *S. aureus, Rhizobium tropici, L. monocytogenes* and *B. subtilis* to convert the negatively charged phosphatidylglycerol (PG) to a positively charged molecule imparting resistance to cationic antimicrobial peptides (CAMPs). In other organisms such as *P. aeruginosa,* a cognate Ala-tRNA^{Ala} (blue) is utilized to neutralize PG conferring resistance to acidic environments. This activity also imparts resistance to chromium, the β-lactam, cefsulodin, and protamine in *P. aeruginosa*.

membrane protein that consists of 13 transmembrane spanning helices and a C-terminal hydrophilic region located in the cytoplasm. In *Staphylococcus aureus* two domains of MprF have been classified [1]. The cytoplasmic region of MprF is responsible for the transferase activity while the membrane spanning region acts as a flippase, allowing modified lipids to reach the outer leaflet of the cell membrane [1].

Background

Homologs of *mprF* are found among diverse bacterial species, both Gram-negative and Gram-positive, as well as in 3 archeons [2]. Bacterial species possessing *mprF* homologs occupy diverse habitats ranging from the soil microorganism *Bacillus subtilis* to the intracellular pathogen *Listeria monocytogenes*. For some organisms the role of *mprF* in bacterial survival is well characterized, such as *S. aureus*, the membrane of which contains a two-component regulatory system that directly senses the presence of CAMPs and upregulates *mprF [3]*. This up-regulation results in increased lysyl-phosphatidylglycerol (K-PG) levels in the membrane and resistance to CAMPs produced by the innate immune system during host colonization [3]. Similarly, the production of K-PG and lysyl-diphosphatidylglycerol in *L. monocytogenes* has been shown to increase resistance to CAMPs and play a role in the entry of the pathogen into gastrointestinal epithelial cells [4]. In *L. monocytogenes* regulation of *mprF* is controlled by the master regulator VirR, which regulates cell wall biosynthesis genes [5]. The role of *mprF* in *Pseudomonas aeruginosa* survival has been extensively characterized by phenotypic microarray (PM), a recent technology that allows many growth conditions to be screened simultaneously [6]. *P. aeruginosa* utilizes a cognate Ala-tRNA^{Ala} to modify PG, allowing

it to resist killing by protamine sulphate, cefsulodin, chromium, sodium lactate, and acidic conditions [6]. MprF also allows the root colonizing *Rhizobium tropici* to resist acidic conditions as well as CAMPs produced by the plant immune system [7].

In *B. subtilis* MprF has been shown to produce K-PG and provide resistance to bacteriocins, or CAMPs, produced by other bacterial species that occupy the same niche [8]. The goal of this research is to use phenotypic microarray technology to determine if MprF impacts an expanded set of phenotypes in *B. subtilis* and correlate these to other characterized phenotypes of bacterial pathogens and plant symbionts. No clear correlation has been drawn to date between the presence of *mprF* among such diverse bacterial species and its role in environmental survival.

Materials and Methods

Strain construction - An I-SceI-mediated deletion method was used to make a clean deletion of *mprF* in *B. subtilis [9]*. The plasmid construct, which contained homologous regions to portions of the genome on either side of *mprF* and an I-SceI restriction site was provided by Kiley Dare. Competent *B. subtilis* 1A100 cells were prepared using modified competence medium (MC) (100mM potassium phosphate pH 7, 3mM trisodium citrate, 3mM $MgSO_4$, 2% glucose, 22 mg/L L-tryptophan, 0.1% casein hydrolysate, 0.2% potassium glutamate). Two culture tubes containing 5ml of MC were inoculated with similarly sized isolated colonies from a streak of *B. subtilis* 1A100 on Luria-Bertani (LB) plates and grown over night at 37 ºC. Tubes were placed in a 37 ºC shaking incubator and OD_{600} readings were taken every 20 minutes of one culture to

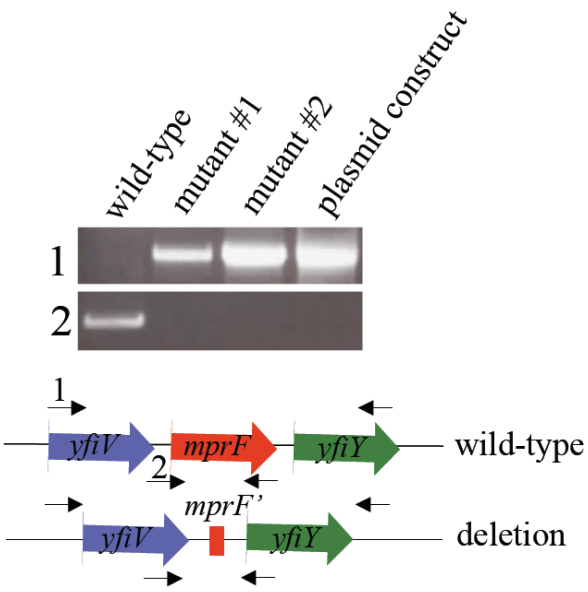

Figure 2. Confirmation of *mprF* deletion in *B. subtilis*. Two sets of primers were used to amplify regions surrounding *mprF* or *mprF* itself. Bands the correct size for amplification of the region surrounding a truncated *mprF* are shown for primer set 1. Only the wild-type produces a band for primer set 2, designed to amplify *mprF* itself and a control for re-localization within the genome.

determine T0, the time in which the cells enter stationary phase. At T90 cells from the duplicate tube, from which no readings were taken, were added to sterile test tubes containing 5 µl of the plasmid construct. These tubes were incubated for an additional hour before 100 µl were removed and plated on LB+spectinomycin (100ug/ml). Single colonies were picked and isolated by streaking twice on LB+spectinomycin (100ug/ml). The competency procedure described above was then repeated with these colonies. The I-SceI encoding plasmid was used to transform these isolates and transformants were selected for on kanamycin (5ug/ml). Clones were isolated by streaking twice and then twice more on LB to allow for loss of the I-SceI encoding plasmid. Final isolates were streaked on LB, LB+spectinomycin (100ug/ml), and LB+kanamycin (5ug/ml) to select for susceptibility to the two antibiotics, which indicated a loss of both the integrated plasmid from the chromosome and loss of the plasmid encoding the I-SceI enzyme. To further test for deletion of *mprF*, polymerase chain reaction was carried out using primers to regions outside of and within *mprF* on genomic DNA from the isolates.

Phenotypic microarray - In order to determine the physiological relevance of *mprF* a Biolog Phenotypic Microarray (PM) was conducted to test just under 2000 phenotypes at once by measuring bacterial growth via an indicator dye that responds to cellular respiration. The PMs of the wild-type versus deletion strains were complied using OmniLog from which the antibiotics Azlocillin and Cefsulodin, both of which target the cell wall, were chosen for further study. Growth curves in the presence of increasing concentrations of both antibiotics were conducted using the OmniLog technology. To prepare the growth curves both the wild type and deletion strains were grown overnight at 37 ˚C on BUG+B agar and then subcultured a second time. From each of the subcultures a cell suspension was prepared in a sterile capped tube containing 20 ml of inoculating fluid to a transmittance of 81%. The cell suspensions, inoculating fluid, dye mix and PM 9+ additive were combined and 50µl were added to each well of a 96-well microtiter plate. A dilution series of Azlocillin (0µl/ml-137.5µl/ml) and Cefsulodin (0µl/ml-27.5µl/ml) was made and added in duplicate to the plate as well.

Results

A successful deletion of *mprF* from *B. subtilis* 1A100 was obtained as illustrated by Figure 2. Comparison of *B. subtilis* Δ*mprF* and *B. subtilis* 1A100 (wild-type) yielded a multitude of phenotypes as seen in Table 1. In the presence of certain compounds the wild-type was able to grow better than the deletion strain suggesting that under these

conditions *mprF* provides resistance. In other conditions the deletion strain grew more robustly indicating the activity of MprF makes the organism more susceptible to the tested compounds. The majority of compounds to which *mprF* provides resistance are those that target the membrane, while the conditions under which *mprF* appears to be harmful to *B. subtilis* include a strong detergent and a toxic anion, in addition to several other compounds. Cefsulodin and Azlocillin were selected to further investigate these phenotypes by testing their effects at several concentrations in the phenotypic microarray assay. These tests showed a trend of increasing susceptibility of the deletion strain to increased concentrations of antibiotic. The data obtained from individual growth curves using these compounds indicated that there is, in fact, a growth phenotype associated with the addition of these two compounds.

Table 1. Phenotypic microarray results from *B. subtilis* wild-type vs. *B. subtilis* Δ*mprF*. *

mprF confers resistance to:

Compound	Description
Antimicrobial Agents	
Naladixic Acid	quinolone, DNA gyrase
Cefsulodin	wall, cephalosporin
Azlocillin	wall, lactam
3,5-Dinitrobenzene	ionophore, respiration
Phenylarsine Oxide	tyrosine phosphatase
Chelerythrine	protein kinase C
Compound 48/80	phospholipase C, ADP ribosylation
Amitriptyline	membrane, transport
Protamine Sulfate	membrane, ATPase
Phleomycin	DNA damage
Coumarin	DNA intercalator
5,7-Dichloro-8-hydroxyquinoline	lipophilic, chelator
5-Chloro-7-Iodo-8-Hydroxyquinoline	lipophilic chelator
Inhibitor	
Phenyl-Methyl-Sulfonyl-Fluoride (PMSF)	protease inhibitor, serine
Orphenadrine	cholinergic antagonist
Detergent	
Domiphen bromide	cationic, fungiside
Antiseptic/Herbicide	
Chloroxylenol	topical antiseptic
Patulin	antifungal, tubulin binding

mprF confers susceptibility to:

Compound	Description
Antimicrobial Agents	
Furaltadone	DNA synthesis, nitro-compound
Diamide	oxidation, glutathione
Sodium Metaborate	transport, toxic anion
Cefotaxime	wall, cephalosporin
Carbenicillin	wall, lactam
Inhibitor	
Lidocaine	ion channel inhibiotr, Na^+
Detergent	
Dodecyltrimethyl Ammonium Bromide	cationic detergent
Antiseptic/Herbicide	
Disulphiram	fungicide

*performed by KD with assistance from BD.

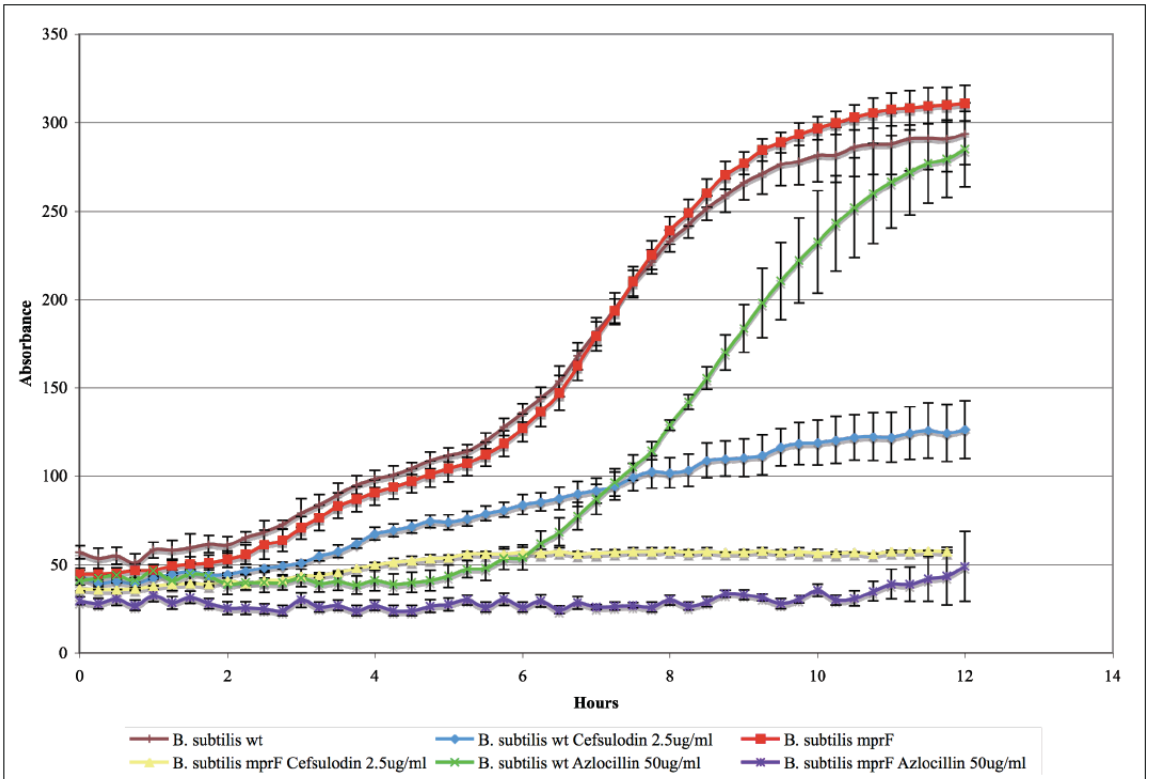

Figure 3. Growth curves of *B. subtilis* wt and *B. subtilis* Δ*mprF* measuring growth by respiration via oxidation of the Biolog redox dye. These graphs represent the optimal concentrations of antibiotic that exhibited the largest growth phenotype. For the antibiotic cefsulodin the optimal concentration is 2.5ug/ml, for azlocillin 50ug/ml.

Discussion

Results from the phenotypic microarray tests described here provide a broader range of effects than reported for similar tests performed on *P. aeruginosa* and *L. monocytogenes* [6](KD unpublished data). In these cases the resistance mediated by *mprF* seems to directly relate to bacterial survival in their host environments. Results obtained from *L. monocytogenes,* seen in Table 2, show resistance to osmolytes such as salt and urea as well as several highly cationic antimicrobial agents. These phenotypes can be easily related to the necessity for survival of *L. monocytogenes* in the host gastrointestinal system where it invades epithelial cells to establish infection. Also, comparison to results obtained from *P. aeruginosa* show *mprF* imparts resistance to

acidity and the CAMP protamine sulphate, which points to a mechanism of survival in the upper respiratory tract. *B. subtilis,* on the other hand, has no clear phenotypic pattern that emerges to pinpoint its reason for possessing *mprF*. It has been shown that *mprF* in *B. subtilis* provides resistance to bacteriocins, but we see an expanded set of phenotypes in this case not limited to bacteriocins.

Table 2. Phenotypic microarray results from *L. monocytogenes* wild-type vs. *L. monocytogenes* Δ*mprF*.*

mprF confers resistance to:		*mprF* confers susceptibility to:	
Compound	**Description**	**Compound**	**Description**
Osmolytes		**Antimicrobial Agents**	
Urea 5%	denaturant	Chloramphenicol	Aminoglycoside, positively charged
NaCl 5%	positively charged ion	**Inhibitor**	
Sodium Sulfate 3%	positively charged ion	orthovanadate	Phosphatase inhibitor, small negatively charged
Potassium Chloride 3%	positively charged ion	**Detergent**	
pH		Niaproof	Anionic surfactant
pH 9.5 + Phenylethylamine	End product of phenylalanine pathway	Dodecyltrimethylammonium brc	cationic detergent
Antimicrobial Agents		**Antiseptic/herbicide**	
Tobramycin	Aminoglycoside, positively charged	Chloroxylenol	topical antiseptic
Geneticin (G418)	Aminoglycoside, positively charged	Pentachlorophenol	insecticide and herbicide
Tannic Acid	Polyphenol, antidiarrheal agent		

*performed by KD.

The key to the phenotypic differences found among these microorganisms is potentially rooted in the reliance of membrane proteins on the composition of the lipid bilayer. Recently, a paper has been published by the Peschel group that shows a change in several proteins localized in the membrane of *S. aureus* in the absence of K-PG [10]. Of interest are members of a membrane stress response regulator as well as several of the proteins under its regulation. Differences in the membrane proteome of other bacteria with expanded phenotypes of *mprF* may show additional differences that explain their phenotype variability. If this is indeed the case an additional global role of K-PG formation in the protein content of the membrane can be characterized. Further insight could be gained by analysis of the membrane proteome of *B. subtilis* in the presence or absence of *mprF* in order to understand how this K-PG synthase effects phospholipid

composition under various stress conditions. The investigation of additional proteins localized to the membrane and their function in the presence or absence of *mprF* will provide information as to how K-PG levels effect other membrane systems in the cell. Initial Biolog PM data showed a marked susceptibility of the *mprF* deletion strain to amitriptyline, the most commonly used tricyclic antidepressant drug that inhibits the re-uptake of noradrenaline and seratonin at nerve endings. This may suggest that K-PG levels interfere with a specific transport or uptake system in *B. subtilis*, indicating a starting point for further studies of the effect of *mprF* on membrane protein function..

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